

Copper-Binding Proteins in Liver of Bluegills Exposed to Increased Soluble Copper under Field and Laboratory Conditions

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Livers from bluegills exposed to increased soluble copper (Cu) under field and laboratory conditions were analyzed to determine the concentration and distribution of Cu in metalloproteins of different molecular size. Analyses were performed on bluegills collected from the impoundment of the H. B. Robinson Steam Electric Plant (Florence, SC) near the effluent discharge from the power plant, near the water intake to the cooling system, and from a control pond as well as on bluegills exposed under controlled laboratory conditions. Metalloproteins were separated into low molecular weight (LMW), intermediate molecular weight (IMW), and high molecular weight (HMW) fractions by using high-performance liquid chromatography.

In the field-exposed bluegills, Cu concentrations in the LMW, IMW, and HMW fractions were highest in bluegills from the discharge site and lowest in those from the control pond. In the laboratory-exposed bluegills, Cu concentrations in the fractions increased with exposure concentration and time. Concentrations of Cu in the LMW protein fraction and pellet of bluegills exposed to 160 $\mu\text{g Cu/L}$ appeared to plateau with long exposure times, whereas those in the HMW fraction continued to increase. Bluegills maintained in 80 $\mu\text{g Cu/L}$ water at pH 5.5 accumulated lower concentrations of Cu in the LMW and pellet fractions and higher amounts in the HMW than in those maintained in 80 $\mu\text{g Cu/L}$ at pH 7.0. Mortality was dependent on exposure concentration and duration and was higher in bluegills maintained in water at pH 5.5 than at pH 7.0.

Introduction

Bluegills (*Lepomis macrochirus*) collected from 1976 to 1978 from the cooling lake impoundment of the H. B. Robinson Steam Electric Plant were found to have decreased reproductive capacity and increased structural abnormalities (1,2). The impoundment is situated on Black Creek, which is a tributary of the Pee Dee River; Black Creek is a typical blackwater stream exhibiting low pH and darkly colored water. Water from this impoundment cools the condensers of both the nuclear and fossil fuel units at H. B. Robinson. We discovered elevated levels of copper in the impoundment from the leaching of copper alloys used in the condensers and found that it was in the more toxic labile forms (3). Because it has been determined that many fish are highly sensitive to copper (4), we speculated that the adverse changes in the bluegill population were attributable to copper.

It is well established that the liver is an important site of accumulation and detoxification of metals and that within liver cells, metals are bound to different classes of proteins. Information is available indicating that cop-

per, cadmium, mercury, and zinc in mammalian livers are detoxified by binding to metallothioneins (5). Metallothioneins and proteins serving a similar function to metallothioneins have been identified in aquatic animals. Most of these proteins have been characterized only by their molecular weight and capacity to bind metals and therefore are referred to as metallothioneinlike proteins, because insufficient data are available to establish that they satisfy the criteria set forth for metallothioneins (6). For convenience, we will subsequently refer to metallothioneinlike proteins in aquatic animals as metallothioneins (MTs), even though the criteria have not been fulfilled for all species.

Most information on MTs in aquatic animals is from research on populations exposed to cadmium under laboratory and field conditions (7,8). Fish have been exposed to mercury, zinc, and copper as well as cadmium (9-37). Experiments to determine the effect of copper exposure on MTs in fish have been performed only on coho salmon (20-22) and rainbow trout (30-32). However, copper binding to MTs has been detected in the eel (23), flounder (9), chum salmon (10), killifish (27), skipjack (33), yellowtail (33), blackbreem (33), and carp (33).

This research was performed to determine the quantities of copper associated with metalloproteins in the

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livers of bluegills exposed to different concentrations of soluble copper under field and laboratory conditions. Also, the effect of pH of the water on the amounts present and the distribution among metalloprotein fractions was assessed.

Materials and Methods

Fish Collection and Maintenance

Field-Exposed Bluegills. Bluegills were collected in May and November 1981 from Christensen's Pond (control: water of low copper concentration), and from the impoundment of the H. B. Robinson Steam Electric Plant near the water intake to the cooling system (water of intermediate copper concentration), and near the effluent discharge from the plant (water of high copper concentration). Bluegills were dissected immediately after collection. Livers were frozen with dry ice and preserved frozen (-70°C). Liver samples from fish collected in May from each site were pooled to form a composite sample; muscle tissue from each fish was analyzed individually. In November, ten fish were collected from each site and only the livers were analyzed; livers of two fish were pooled for a total of five samples from each site.

Laboratory-Exposed Bluegills. Bluegills collected by seine from a pond near Sebastopol, CA, were transported to Lawrence Livermore National Laboratory where they were distributed randomly among six 760-L fiberglass bioassay tanks; each tank received 70 fish. Water delivered to the bioassay system was filtered through activated charcoal to remove chlorine, was low in total dissolved solids, and was fully saturated with oxygen. Water flowed through each tank at 1.5 L/min; 90% replacement required 24 hr.

Bluegills were acclimated before exposure to copper. During their recovery from the trauma of transportation, they were conditioned to laboratory food. They were fed brine shrimp as a transition food and then commercial fish food (Oregon moist). They were fed *ad libitum*, twice daily Monday through Friday, and once daily on the weekends. The fish were acclimated to a lower pH water by reducing the pH with HCl by 0.1 pH unit each day.

Young bluegills (6–8 cm) were exposed to water regulated to pH 5.5 and containing either ~ 1 (control), 20, 40, 80, or 160 $\mu\text{g Cu/L}$; in one tank the pH in the water was regulated to pH 7 and the copper at 80 $\mu\text{g/L}$.

Six bluegills were sacrificed from each bioassay tank at 3- to 4-wk intervals over a total exposure period of 15 wk. The bluegills were dissected immediately upon removal from the bioassay tank, and all dissections were performed on ice. The livers from the six fish from each bioassay tank were pooled for metalloprotein isolation.

Isolation of Metalloproteins

Livers were placed in three volumes per weight of nitrogen-saturated, 50 mM Tris-HCl (pH 7.6) containing 10% sucrose, 1% 2-mercaptoethanol, and 200 K.I.U. of Trasylol and homogenized on ice with a polytron. Dupli-

cate aliquots of each homogenate were reserved for metal analysis. Homogenates were centrifuged at 115,000g for 90 min at 4°C . The final clear supernatant fluids (S-115s) were saturated with $\text{N}_2(\text{g})$, frozen immediately with liquid nitrogen, and stored at -70°C until they were chromatographed. Duplicate aliquots of each S-115 and the pellet were reserved for metal analyses.

Aliquots of each S-115 of the livers were processed on a Waters high performance liquid chromatograph (HPLC) fitted with a Varian TSK 3000 SW gel-permeation column ($22 \times 300 \text{ mm}$) that had been calibrated with proteins of known molecular weight. The mobile phase consisted of 0.15 M NaCl Tris-HCl buffer mixture (pH 7.6 at 25°C) at 4 mL/min; fractions were collected every minute for 40 min. Molecular absorbance at 280 nm was monitored continuously, and the fractions collected were analyzed for copper.

Quantities of copper present in four fractions were determined. An insoluble one was recovered as the pellet from the 115,000g centrifugation. The three soluble ones in the supernatant fluid were resolved by HPLC into a low molecular weight (LMW) fraction in the 6,000- to 40,000-dalton range that eluted between 73 and 93 mL, an intermediate molecular weight (IMW) fraction in the 40,000- to 126,000-dalton range that eluted between 61 and 72 mL, and a high molecular weight (HMW) fraction in the $>126,000$ -dalton range that eluted between 35 and 60 mL.

Metal Analyses

Samples for metal analysis were either (1) dried at 100°C , ashed at 450°C , dissolved in a mixture of concentrated HCl and HNO_3 (3:1), and brought to final volume with doubly distilled water or (2) digested with a perchloric and nitric acid mixture and then brought to final volume with double-distilled water. A standard reference material of oyster tissue obtained from the National Bureau of Standards was analyzed with the samples in order to validate analytical procedures. Unknowns and reference material were analyzed with a Perkin-Elmer Model 603 atomic absorption spectrophotometer; measurements were corrected for reagent blanks.

Results

Tissue Metal Concentrations

Copper concentrations in muscle tissue from bluegills collected in May 1981 were lower in fish from Christensen's Pond (control site) than in those from the intake and discharge sites (Table 1). Copper concentrations in liver tissue differed greatly with the collection site. The concentrations in the pooled liver samples from animals collected in May were similar to those collected in November. Copper was lowest in livers from fish from the control pond and highest in those from the discharge site.

Metalloproteins

Field-Exposed Bluegills. Quantities of copper eluted in the HMW, IMW, and LMW fractions and pres-

Table 1. Mean concentrations of metals in tissues of bluegills.

Collection site	Collection date	Tissue	Number of fish	Metal, $\mu\text{g/g}$ dry weight ^a		
				Copper	Cadmium	Zinc
Christenson's Pond	May 1981	Muscle	11	1.51 ± 0.26	— ^b	—
	May 1981	Liver ^c	11	11.3	—	—
	November 1981	Liver	10	13.0 ± 5.7	$<0.15 \pm 0.07$	55.9 ± 6.7
H.B. Robinson, Intake	May 1981	Muscle	37	2.35 ± 0.44	—	—
	May 1981	Liver ^c	37	168	—	—
	November 1981	Liver	10	69.3 ± 38.6	3.58 ± 0.07	61.2 ± 6.7
H.B. Robinson, Discharge	May 1981	Muscle	42	2.16 ± 0.37	—	—
	May 1981	Liver ^c	42	558	—	—
	November 1981	Liver	10	356 ± 200	0.72 ± 0.39	77.0 ± 6.7

^aMean \pm one standard deviation.^bNo analysis performed.^cLivers pooled for single analysis.

ent in the pellet were higher in fish from the intake and discharge sites than from the control sites. Elution profiles of copper in S-115s of liver tissue of the two bluegills that had the highest concentration of copper at each site are shown in Figure 1a. The mean of the copper concentrations in the LMW fraction in S-115s from fish from the discharge site was 240 ± 120 $\mu\text{mole/kg}$ wet tissue compared to 13 ± 7 $\mu\text{mole/kg}$ from the control site; the mean from the intake site was 79 ± 28 $\mu\text{mole/kg}$. Large differences were found also in the HMW fraction; the mean from fish from the discharge site was 62 ± 52 $\mu\text{mole/kg}$, from the intake site was 5 ± 3 $\mu\text{mole/kg}$, and from the control site was 2 ± 0.3 $\mu\text{mole/kg}$ wet tissue.

The quantities of Zn present in the HMW, IMW, and

LMW fractions were smallest in S-115s from the discharge site and largest in those from the control site. Elution profiles of zinc are shown in Figure 1b. Elution profiles for Cd were not constructed because Cd concentrations in the fractions from the HPLC column were below detection limits for almost all fractions. An ultra-violet absorbance profile characteristic of all HPLC chromatograms is shown in Figure 1c.

The relationships between the copper concentration in the liver and those in the LMW, IMW, HMW, and pellet fractions were determined. In fish from the control and intake sites, the quantities of copper eluted totally and in the pellet were directly related to the copper concentrations in the liver. The equation of the line for the total

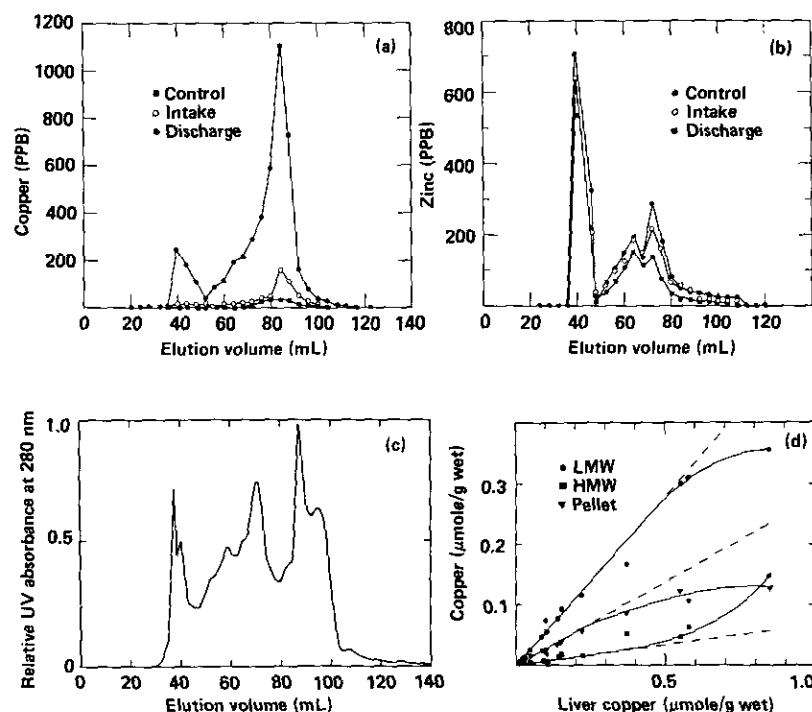


FIGURE 1. Field-exposed bluegills. Metal and ultraviolet absorbance elution profiles: (a) elution profiles of Cu in the S-115s of liver tissue of the bluegills that had the highest concentration of copper at each site; (b) elution profiles of Zn in S-115 of liver tissue of the two bluegills that had the highest concentration of Cu at each site; (c) characteristic UV absorbance profile at 280 nm of S-115s of liver tissue of bluegills from the three collection sites; (d) concentrations of Cu in the LMW, HMW, and pellet fractions compared to that in the liver.

eluted versus liver copper was $y = 0.76x + 7.1$; R^2 was 0.98; the equation for the line for the pellet was $y = 0.29x + 13.6$; R^2 was 0.99. For this same group of fish (control and intake), the quantities in the LMW, IMW, and HMW fractions were directly related to the total eluted. The equation for the line for LMW fraction versus total copper eluted was $y = 0.73x - 11.3$; R^2 was 1.0. The equations for the IMW and HMW fractions versus total copper eluted were $y = 0.12x - 13$ and $y = 0.1x + 2.5$, respectively; R^2 was 0.89 and 0.97, respectively. The slope of the line for the LMW fraction was about seven times greater than that for the HMW fraction. These data indicate that in livers of the intake and control fish, the LMW fraction containing the MTs is by far the largest depot of copper. However, increases in soluble copper in the liver result not only in increases in the LMW fraction, but also in increases in the IMW and HMW fractions.

At the high concentrations measured in the livers of most discharge fish, there was a departure from the linear relationship between the quantity of copper in each fraction and in the total liver. In the HMW fraction, the quantities were greater, and in the LMW fraction and pellet they were less than expected, relative to those predicted from the copper concentrations in the livers of the control and intake bluegills (Fig. 1d). These data indicate that the capacity to bind or sequester metals of the proteins in the LMW fraction and constituents in the pellet may have been saturated with resultant increases in the quantities partitioned into the HMW fraction.

Laboratory-Exposed Bluegills. In the LMW, IMW, and HMW protein fractions of livers from fish exposed to the different concentrations of copper for 4, 8, 12, and 15 weeks, the quantities of copper increased with exposure time (Fig. 2). Changes with exposure time in the LMW and HMW protein fractions appear linear except for the fishes exposed to 160 $\mu\text{g Cu/L}$. Changes with exposure time in the IMW protein fraction were different from those in the HMW and LMW protein fractions; the changes in fish exposed to 160 $\mu\text{g Cu/L}$ also appeared linear. In the pellets, there was little change with time after the first sampling period except in the group of bluegills exposed to 160 $\mu\text{g Cu/L}$.

The quantities of copper in the different compartments of the liver in fish exposed to 80 $\mu\text{g Cu/L}$ at pH 5.5 were not the same as in those exposed to 80 $\mu\text{g Cu/L}$ at pH 7 (Fig. 3). In fish exposed to copper in water at pH 7, higher levels of copper were reached in the LMW protein fractions and in the pellet; whereas in those exposed at pH 5.5, higher levels were reached in the HMW protein fraction.

The quantities in the LMW and HMW protein fractions appeared directly related to the copper concentrations in livers from bluegills that had liver concentrations $> 1 \mu\text{mole Cu/g wet weight}$ (Fig. 4). However, the slope of the line was much greater for the LMW than the HMW protein fractions. In fish with $> 1 \mu\text{mole Cu/g wet weight}$, quantities in the HMW protein fractions were greater and in the LMW protein fraction were less than expected relative to those indicated from the extension

of the line obtained from data from fish with lower liver concentrations; copper in the LMW protein fraction appeared to plateau, whereas that in the HMW protein fractions appeared to increase.

An increase in mortality was found in all groups of fish (Fig. 5). This mortality was related to dose and time. The increase in rate of mortality after day 70 is partly attributed to decreased temperatures in the tanks because of changes in climatic conditions. These bioassays were conducted in our outdoor exposure facilities. As a result of an unusual period of prolonged cold weather, the temperature in the tanks fell below 13 to 14°C from day 70 until day 105. Though these temperatures are within the normal tolerance range of bluegills, the synergistic effects of temperature with low pH and copper exposure accelerated the death rate.

Discussion

There is considerable information on copper concentrations in tissues of freshwater fish (38). In general, the data support our results and indicate that muscle tissues contain low concentrations of copper and do not reflect increases of copper in the environment, whereas liver tissues are high in copper and do reflect such changes.

Liver copper concentrations have been determined for bluegills from different sites (2,39,40). Data obtained by Benoit are of special interest because the analyses were performed on fish exposed chronically in the laboratory to known concentrations of copper (39). Although he found that the copper concentrations in the livers of fish exposed to 12, 21, 40, 77, and 162 $\mu\text{g Cu/L}$ increased with exposure concentrations, only the difference between those exposed to 162 $\mu\text{g Cu/L}$ and controls were significant because of the large variability within each sample group. Variability appeared to increase with exposure concentration; the fractional standard deviation of the mean copper concentration was higher in copper-exposed than in control fish. These data indicate that there are large differences in response of fish to the same concentration of copper.

Our data on metalloproteins, like that of others, support the concept that MTs play a role in the detoxification of metals. We found much higher concentrations of copper in the LMW protein fraction from livers of bluegills exposed to increased soluble copper in both the field and laboratory than in those that had not been exposed. Our data for bluegills exposed chronically to known concentrations of copper in the laboratory indicate that the quantities of copper associated with the LMW protein fraction, which contains the MTs, are related to exposure concentration and duration. Our results are similar to those obtained in fish exposed to cadmium (12,15) and to copper (20,22,30-32).

Proteins in the LMW fraction in the bluegill livers appear to bind copper, cadmium, and zinc. We have no information on the relative binding affinities for the three metals in bluegills. Noël-Lambot et al. found that zinc, and not copper, could be displaced by cadmium on the MTs in liver of eels (23). Our data indicate that Cu may

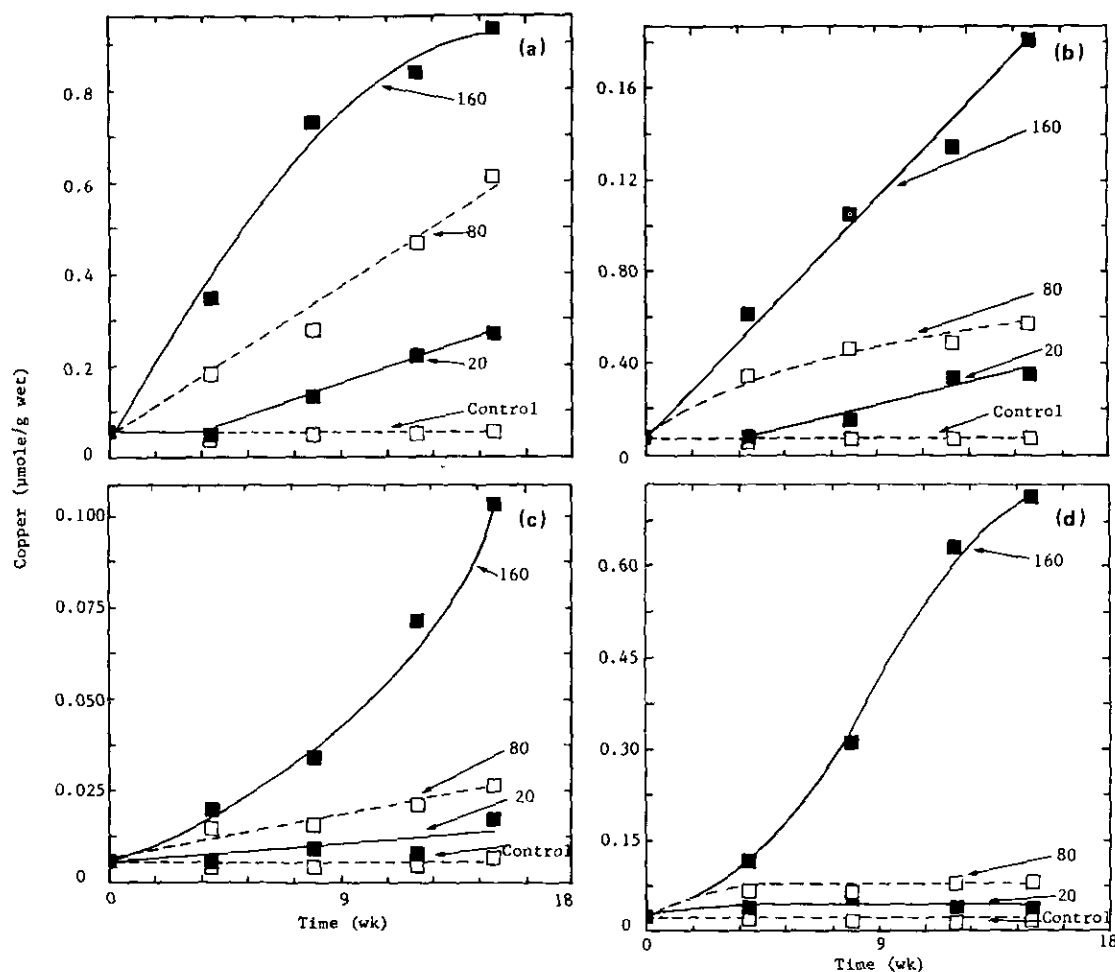


FIGURE 2. Laboratory-exposed bluegills. Changes with time in the concentration of Cu in different fractions of liver tissue; (a) LMW protein fraction of S-115s; (b) HMW protein fraction of S-115s; (c) IMW protein fraction of S-115s; (d) pellet fraction of liver tissue homogenate.

have displaced Zn from proteins in the LMW fraction in livers from fish from the discharge site.

Limited data are available on the changes that occur with time in the quantities of metals bound to MTs even though in many ecosystems fish are subjected to chronic exposure to low levels of metals. The changes with exposure time in the quantities of Cu in the LMW fraction of the supernatant fluid from the group of bluegills exposed to 160 $\mu\text{g Cu/L}$ indicated that the amount of Cu associated with the MTs was reaching a plateau. These data provide indirect evidence that the quantity of MT available for metal binding is limited.

Direct evidence was obtained by McCarter and Roch (21), who measured the concentration of MTs in the livers of juvenile coho salmon that had been exposed to about 1, 50, 100, and 150 $\mu\text{g Cu/L}$. They found that the concentration of MT in the livers of fish exposed to increased soluble copper increased as a function of time to a maximum at 4 weeks exposure and thereafter remained more or less constant. However, the concentration of MT at this time was related to the concentration of Cu to which the fish had been exposed. Limitations in the amount of Cu

associated with MT could result because the quantities of MTs that can be synthesized are genetically controlled to a prescribed level or because the increased quantities of Cu in the cytosol interfere with the synthesis of additional MTs or with a metabolic process that is critical for the synthesis of MTs.

The distribution of copper among the LMW, IMW, and HMW protein fractions as well as the pellet was different in bluegills exposed to Cu at pH 5.5 and 7.0. At the lower pH, there were greater quantities of copper associated with the HMW protein fractions than at the higher pH. Consequently, in the fish in water of lower pH, the metalloenzymes that are present in this fraction may have a greater potential for being competitively bound to copper.

Information on the distribution of copper in different fractions of the liver as a function of liver copper concentration is limited. A number of investigators have measured copper concentrations in LMW and HMW protein fractions of the cytosol of fish livers, but have not related them to total liver concentrations. We measured the quantities of copper not only in the LMW, IMW, and

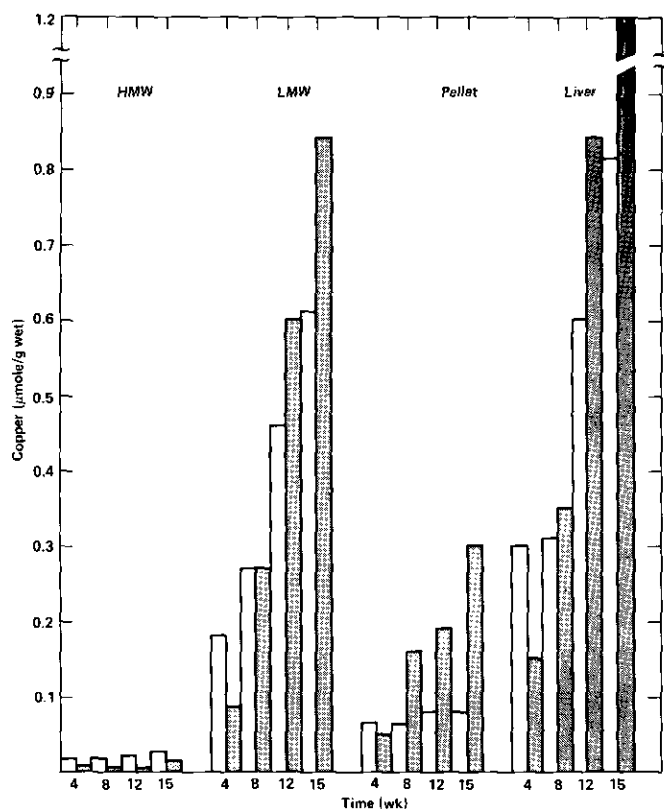


FIGURE 3. Quantities of Cu in the different fractions of livers. Bluegills were exposed to 80 $\mu\text{g Cu/L}$ (open bars) at pH 5.5 and (shaded bars) at pH 7.0.

HMW protein fractions, but also in the pellet (insoluble fraction of the liver). Although we did not identify the constituents in the pellet containing the Cu, information from other studies indicates that they may contain membrane-bound vesicles that accumulate copper (7, 41-45).

Our data on the relationship between the quantities of Cu in the four fractions and those in the liver indicate that the quantities of Cu in the LMW protein fraction were approaching a plateau, whereas those in the HMW protein fraction were continuing to increase. A similar relationship between quantities of these same fractions in the digestive gland (analogous to liver tissue) has been shown for the mussel *Mytilus edulis* (46). It is interesting that Benoit (39) found the group of bluegills that showed decreased reproductive capacity and increased mortality in his studies had about the same total liver Cu concentrations as the bluegills in our studies; these bluegills had quantities of Cu in the LMW fraction that were in the region of the plateau.

Our data on distribution of copper indicates that in bluegills having liver Cu concentrations $< 1 \mu\text{mole Cu/g wet weight}$, the quantities of Cu in the LMW and HMW protein fractions were directly related to the total quantities of copper in the liver. Also, in livers that contained $< 1 \mu\text{mole Cu/g wet weight}$, the slope of the line was much greater for the LMW fraction than for the other fractions. We propose that in animals having relatively

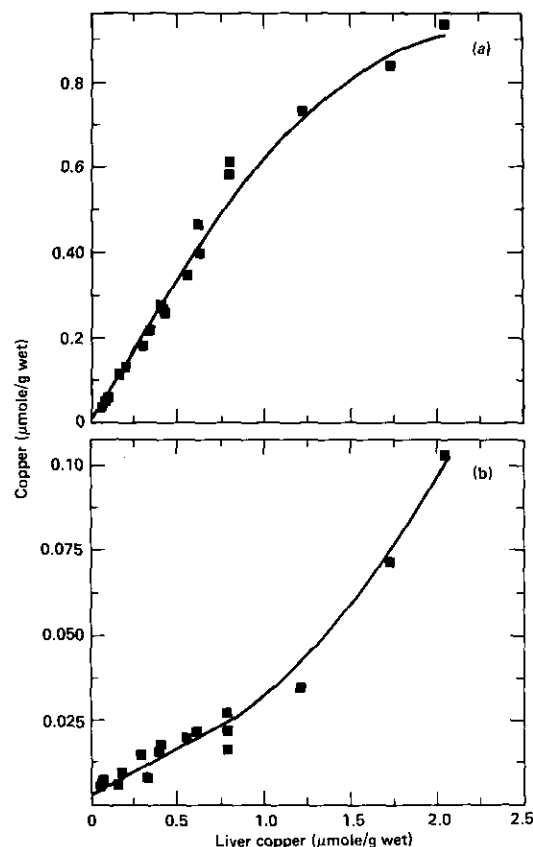


FIGURE 4. Concentrations of Cu in the protein fractions compared to that in the livers of bluegills exposed to different concentrations of Cu: (a) LMW protein fraction; (b) HMW protein fraction.

low concentrations of Cu in the liver (in bluegills $< 1 \mu\text{mole Cu/g wet tissue}$), Cu reaching the cells is partitioned among the compartments on the basis of the relative affinity for copper of the constituents in the fractions. The detoxification and/or homeostatic action provided by the MTs appears to be afforded by its higher affinity for Cu and/or its higher concentration.

Deviation from a linear relationship between the amount in a fraction and that in the liver appeared to occur in bluegills with concentrations of Cu in the liver $> 1 \mu\text{mole Cu/g wet weight}$. The causal factors were not identified, but may be related to the relative rates of synthesis and degradation of the molecules in the fractions that bind Cu. Some information is available on the rate of turnover of MTs. McCarter and Roch (22) determined the half-life for loss of MTs in juvenile coho salmon to be a function of the exposure concentration of Cu. For control fish, the half-life was 13 days, and for fish exposed to 100 $\mu\text{g Cu/L}$ for about 8 weeks, it was 30 days.

Deleterious effects of metals are related not only to current but also to past exposure conditions. Considerable evidence is available indicating that fish pre-exposed to sublethal doses of metals are more tolerant of metals in subsequent exposures (12, 17, 21, 47-50). For a few species, this increased tolerance has been related directly to the concentration of MTs (17, 21). Information about increased tolerance of aquatic organisms related

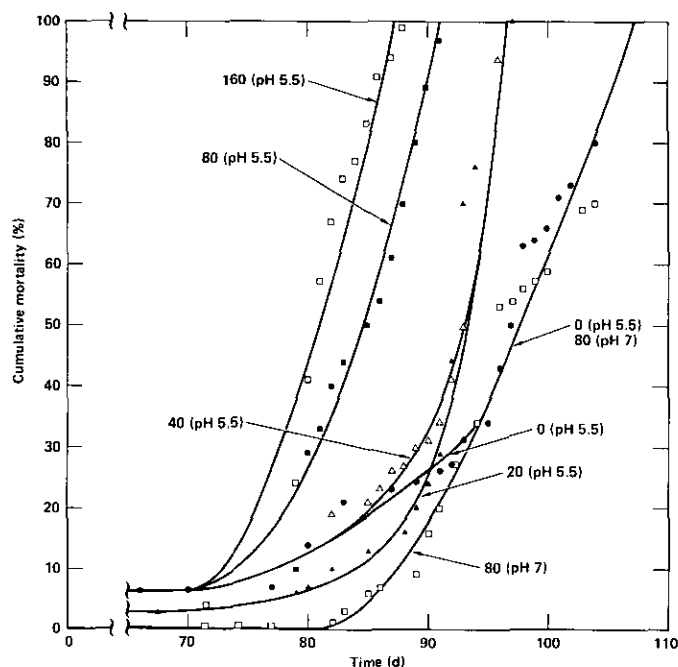


FIGURE 5. Cumulative mortalities of bluegills exposed to Cu under controlled laboratory conditions. Exposure concentrations ($\mu\text{g Cu/L}$) are adjacent to the curves. Low temperatures in the bioassay water initiated at about the 70th day of exposure.

to the presence of increased quantities of MTs becomes important for predicting the capacity of ecosystems to receive additional quantities of metals, and for setting standards that protect the quality of the environment. Because of the high level of sensitivity of early life stages of fish to metals, it is especially important to have data on the potential for enhanced tolerance in these stages to increased metal concentrations.

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